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THE IDENTIFICATION OF A PHOSPHOPROTEIN IN ACANTHOLYTIC EPIDERMIS*

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It has been shown previously that drugs which inhibit respiration and glycolysis also inhibit cantharidin-induced acantholysis (1–4). From this it was inferred that acantholysis requires an energy source, probably with ATP as an intermediate (4). The present communication deals with the fate of cellular phosphate in epidermal slices during acantholysis. It was found that slices which undergo extensive acantholysis in the presence of disodium cantharidin contain up to 12 times the phosphoprotein content of control slices. Investigation of acid and enzymic hydrolysates of the phosphoproteins indicated that the phosphate is covalently bound as O-phosphoserine and, thus, probably requires ATP for its formation.

MATERIALS AND METHODS

Carrier-free inorganic phosphate-^{32}P was diluted with unlabeled sodium phosphate and was purified according to Conover and associates (5). This was used in the preparation of Krebs-Ringer phosphate buffer, pH 7.2, which contained approximately 1.0 μc of ^{32}P per ml for most studies. For the isolation and characterization of phosphoserine, the radioactivity was increased to 100 μc of ^{32}P per ml of buffer. Slices were incubated in 1.5 ml of a medium containing 1 ml of the Krebs-Ringer solution, 10 μmoles of glucose, 5 μmoles of tris at pH 7.2, and 50 μg of chloramphenicol.

Most of the studies were made with epidermal slices from beef snout obtained from animals slaughtered on the morning of the experiment. A few studies were made with 0.2 mm slices of human skin obtained with a keratotome at autopsy or with whole skin from the hairless mouse. When beef snout was used, the first 0.2 mm slice (consisting of stratum corneum) was discarded. The second slice, 0.3 mm thick and representing mainly the prickle cell layer (Fig. 1 *left*) was cut into specimens of 50 to 100 mg and weighed immediately. Specimens were usually incubated for 3 hours with cantharidin without preincubation.

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For the distribution studies, tissues were preincubated in medium for 30 minutes before addition of isotonic saline or 1.5 μmoles of disodium cantharidin (DSC); the incubations were continued for 90 minutes. After incubation, tissues were rinsed three times in saline and were homogenized in 1.5 ml of 6M urea with ground-glass homogenizers. Centrifugation of the homogenates at 10,000 × g for 10 minutes removed a small amount of heavily pigmented residue which contained little radioactivity and was discarded. The resulting supernates were used for subsequent studies.

Analytical Methods. Radioactivity was counted, on a Packard Tri-Carb liquid scintillation spectrometer, in aliquots of fractions dispersed in Diotol (6). Counts were corrected for decay of the ^{32}P and for quenching (internal standards).

O-Phosphoserine was detected in fractions from the Dowex 50 column and on paper with ninhydrin.

Inorganic phosphate from the column was measured by the method of Gomori (7). Assay for the phosphate of the phosphoprotein required the following adaptation of a procedure of Martin and Doty (8). A lipid-free protein residue was incubated overnight in 1 ml of 1N NaOH at 37° C. The soluble proteins and peptides were removed with 0.4 ml of 0.02M silicotungstic acid after acidifying the solution with 3.0 mmoles of perchloric acid. Aliquots of the supernate were mixed thoroughly with 0.05 ml of 5M H_2SO_4, 0.5 ml of H_2O, 0.05 ml of 10% ammonium molybdate, and 1 ml of isobutanol-benzene, 1:1. To 0.5 ml of the clear upper phase were added 0.5 ml of 3.2% H_2SO_4 in ethanol and 0.05 ml of fresh 0.05% stannous chloride. The absorbance of the solution was read immediately at 700 mμ in microcuvettes. It is necessary to use acid-washed glassware for this determination because ordinary procedures of glasswashing leave a small amount of phosphate which is enough to produce erratic results.

RESULTS

Distribution of Phosphate-^{32}P in Acantholytic Epidermis. The clear supernates of the urea extract were diluted with 3 volumes of water and were acidified with 3 mmoles of perchloric acid to precipitate proteins. Inorganic and organic phosphates were separated from aliquots of the resulting supernates by the modified method of Berenblum and Chain (9). Phospholipids were extracted from the residue with two washes with chloroform-methanol, 2:1

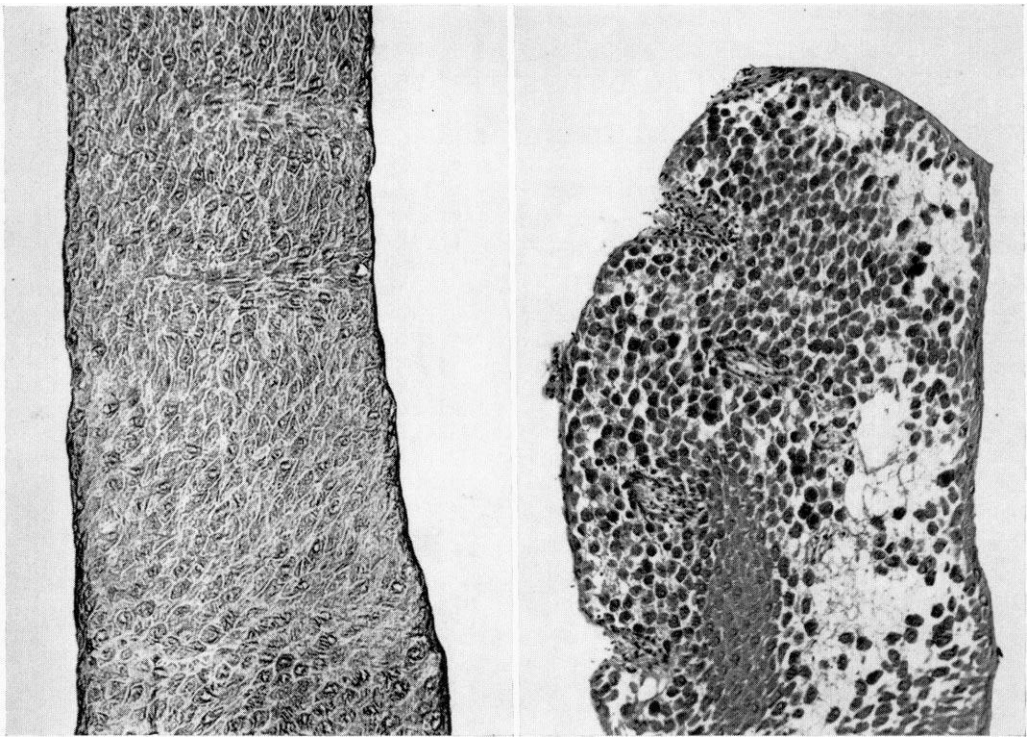

FIG. 1. Acantholysis in beef snout epidermis. *Left,* Specimen (4 mm²) incubated for 90 minutes in Krebs-Ringer phosphate buffer. (Hematoxylin and eosin; × 140). *Right,* Specimen incubated for 90 minutes in Krebs-Ringer phosphate buffer containing 5×10^{-4}M cantharidin. (Hematoxylin and eosin; × 110.)

TABLE I

Effect of cantharidin-induced acantholysis on distribution of phosphate-^{32}P in slices of beef snout epidermis

Fraction	Total counts per minute per 10 mg of tissue, wet weight (mean ± SD, 12 experiments)	
	Control	Cantharidin-treated*
Acid-soluble	5,461	4,620
Inorganic	3,387 ± 175	2,700 ± 151
Organic	2,074 ± 146	1,920 ± 108
Residue	347.8	1,133.7
Lipid	60.4 ± 3.9	75.0 ± 6.5
DNA	7.3 ± 1.8	9.1 ± 2.4
RNA	53.8 ± 10.6	80.6 ± 10.1
Protein	226.3 ± 7.3	969.0 ± 65.0
Overall total	5,809	5,754

* Disodium cantharidin, 1mM.

(10), and deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and phosphoproteins were fractionated according to the procedure of Schmidt and Thannhauser (11).

TABLE II

Effect of acantholysis on distribution of phosphate-^{32}P in epidermis (human and hairless mouse)

Fraction	Total counts per minute per 10 mg of tissue, wet weight			
	Human		Hairless mouse	
	Control	DSC-treated	Control	DSC-treated
Acid-soluble esters	7,380	4,110	12,950	11,590
Lipid	160	158	305	506
Residue	428	827	256	1,053

A significant redistribution of cellular phosphate-^{32}P occurred in epidermal specimens after acantholysis. The radioactivity in the residue from bovine slices increased by 226%, and similar but quantitatively different increases occurred in the residual fractions of epidermis from humans and hairless mice (Tables I and II).

TABLE III

Effect of acantholysis on specific radioactivity of epidermal phosphoprotein

Tissue	Samples (no.)	P (mμ-moles/mg)	^{32}P (cpm/mg)	Specific activity
Control	4	0.95	11	11.6
Acantholysis	4	5.3	136	25.8
Ratio: acantholysis/control		5.6	12.4	0.45

TABLE IV

Distribution of phosphate-^{32}P in fractions resulting from enzymic and acid hydrolysis of perchloric acid-precipitated residue

Fraction	Total ^{32}P (cpm)
Whole residue	15,015
Supernate after enzyme hydrolysis	14,652*
Organic phosphate	13,488
Inorganic phosphate	1,164
Supernate after acid hydrolysis	12,450*
Organic phosphate	8,550
Inorganic phosphate	3,900

* Calculated as the sum of organic and inorganic phosphate.

The major portion of the labeled phosphate of the residue from bovine slices had the properties of protein-bound covalent phosphate as indicated by the data below. The radioactivity associated with the protein increased by 330% after acantholysis and constituted 17% of the total phosphate-^{32}P in the cell. Counts were increased slightly in the nucleic acid and phospholipid fractions also but, because fractionation by the procedure of Schmidt and Thannhauser may not be complete (12), this is probably the result of contamination from the large amount of phosphate-^{32}P in the protein fraction.

Chemical measurement of phosphate from the phosphoprotein produced in 3 hours of incubation showed a 5.6-fold increase in total protein-bound phosphate with acantholysis (Table III). The radioactivity of the phosphate in these experiments increased 12-fold, resulting in a doubling of the specific activity. The protein-bound ^{32}P was 30% of the total cellular ^{32}P.

Accompanying the increase in protein-bound phosphate-^{32}P was a 15% decrease in acid-soluble phosphate-^{32}P (Table I). The decrease was attributed to both organic and inorganic phosphates, although the greatest change was in the latter. Total cellular phosphate-^{32}P remained unchanged.

Nature of the Residue-Phosphate Complex. The major portion of the labeled phosphate from cantharidin-treated specimens behaved like phosphoprotein. Inorganic phosphate-^{32}P was liberated when the residue was incubated in 1N alkali for 18 hours at 37° C, but the complex was stable in 0.04N alkali at 4° C for at least 12 hours, during which time it dissolved. It was stable also to 6M urea, dialysis, hot ethanol, and 5% trichloroacetic acid at 90°C. During isolation of phosphoserine (below) it was found that incubation of the residue with two proteolytic enzymes solubilized most of the complex (Table IV). Ninety-two per cent of the phosphate-^{32}P was found to have the solubility characteristics of organic phosphate, suggesting that it was covalently bound to an amino acid or peptide derived from the original proteins. Acid hydrolysis resulted in conversion of 20% of this covalent phosphate to inorganic phosphate. When a urea extract of an acantholytic specimen was dialyzed overnight against water, protein precipitated; this was redissolved in cold 0.04N NaOH. While the pH was monitored, the solution was titrated with dilute acid, and the protein was found to precipitate in at least two fractions, with the radioactivity being distributed about equally between fractions precipitating at pH 6.3 and 5.5. None of the first residue and about 20% of the second residue could be dissolved in 0.1M citric acid buffer, pH 2.5 (13).

Separation and Identification of Phosphoserine in Hydrolysates. A specimen of snout was homogenized in urea after incubation with DSC for 3 hours in the high-activity buffer. The supernate after centrifugation was diluted with water, and the proteins were precipitated with perchloric acid as before; phospholipids were extracted from the residue. Phosphoserine was released from the residue and identified essentially according to the method of Heald (14), but preliminary digestions with two proteolytic enzymes were used to reduce the time necessary for acid hydrolysis, since considerable phosphoserine may be destroyed during acid

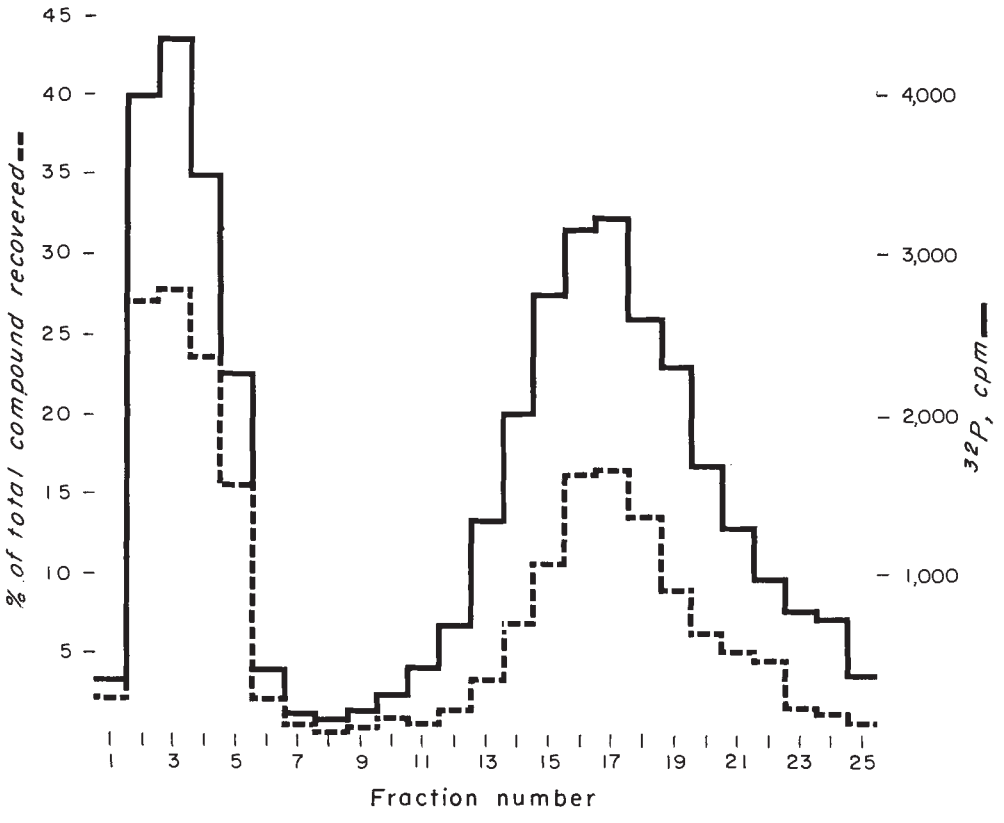

FIG. 2. Fractionation of acid hydrolysate of phosphoprotein on Dowex 50 × 8, 200–400 mesh, 1 by 13 cm. Eluant, 0.05N HCl; fraction volume, 2.0 ml. Broken line shows percentage of total compound recovered; solid line shows radioactivity as ^{32}P, counts/min. Peak 1 = inorganic phosphate. Peak 2 = phosphoserine.

hydrolysis. The lipid-free residue was homogenized in 2 ml of tris buffer, pH 7.5, containing 5 mg of trypsin and was incubated for 4 hours at 37°C; this was followed by another incubation with 5 mg of pepsin for 4 hours at pH 2.6. The supernate from these incubations was made 2N in HCl and was digested at 120°C for 8 hours. The hydrolysate was filtered, 20 μ-moles of inorganic phosphate and 4 μmoles of *DL*-O-phosphoserine were added, and the solution was taken to dryness in vacuo to remove the HCl. The inorganic phosphate and phosphoserine were fractionated on a 1 by 13-cm column of Dowex 50 × 8 (14). The eluate which corresponded to phosphoserine was taken to dryness in vacuo, and a portion was chromatographed on Whatman No. 1 paper in two dimensions (solvent 1, methanol:water:pyridine, 120:30:6; solvent 2, absolute ethanol:0.1 M acetate pH 4.6, 75:50). The paper was dried and sprayed with ninhydrin and was exposed to no-screen x-ray film for 2 weeks.

Chromatography of the acid hydrolysate on Dowex 50 (Fig. 2) indicated that most of the covalent phosphate-^{32}P was eluted with O-phosphoserine. Rechromatography of the phosphoserine peak on paper revealed that 93% of the radioactivity coincided with the ninhydrin-positive spot of phosphoserine at R_f values of 0.52 and 0.61 in solvents 1 and 2, respectively. Since the incorporation of ^{32}P into control epidermis was so low, it was not determined if phosphate is bound to serine residues of normal epidermal protein.

DISCUSSION

Numerous seemingly unrelated agents and conditions cause a separation of epidermial cells resembling acantholysis. Do these agents and conditions produce a common structural change in the epidermal cell? If they do, what is the change? Proteolytic enzymes, thiol compounds, heat, and urea all are known to cause acantholysis (15) and all produce changes

in proteins. Although evidence for protein changes in cantharidin-induced acantholysis has been sought, no good proof has heretofore been found. Thus, the finding of increased quantities of phosphate-^{32}P in the protein fraction from epidermis treated with cantharidin stimulated interest because it seemed to be the first evidence that such a change is taking place—a change of an epidermal protein to a phosphoprotein.

However, increased phosphate-^{32}P content in a protein fraction does not, of itself, prove that net phosphoprotein synthesis has occurred. It is often difficult to remove small amounts of inorganic phosphate-^{32}P from certain protein fractions, particularly proteins of a fibrous nature, apparently because it becomes physically trapped (16). It was necessary to show that the phosphate-^{32}P was bound covalently to the protein. When the protein-phosphate-^{32}P complex was treated with proteolytic enzymes it yielded acid-soluble peptides or amino acids containing phosphate-^{32}P which could not be extracted as inorganic phosphate by the Berenblum-Chain procedure. Furthermore, chromatography of an acid hydrolysate of the phosphoprotein residuum indicated that 90% of the organic fraction was O-phosphoserine. Because the solubility characteristics of the residuum make it unlikely that this phosphoserine was released from phospholipid, the labeled phosphate most likely was bound covalently to protein.

One would observe an increase in ^{32}P-labeled phosphoprotein, but without a net increase in total phosphoprotein, if labeled phosphate merely exchanged with the unlabeled phosphate of phosphoprotein that was present in the cell prior to incubation. This was ruled out by data obtained by chemical determination of phosphoprotein phosphate: a fourfold to sixfold increase in phosphoprotein phosphate was found in the acantholytic protein fractions. The difference between specific activities of the protein-bound phosphate from control slices and from acantholytic tissue could mean that a low rate of exchange was also occurring, but what is more likely is that phosphoprotein existed in the tissue prior to incubation; this would dilute the small amount of new ^{32}P-labeled phosphoprotein from controls more than the large amounts of new ^{32}P-labeled phosphoprotein produced during acantholysis.

The phosphoprotein from the residuum was heterogeneous, but there are some data to suggest that phosphorylation occurred on proteins of structural nature. The protein-phosphate complex was water insoluble, could be dissolved in cold 0.04N NaOH without appreciable decomposition, and contained at least two proteins, precipitating at pH 6.3 and 5.5, similar to the proteins of Crounse (17) and Matoltsy (13). However, the bulk of the protein carrying the phosphate could not be solubilized in citrate at pH 2.6, unlike prekeratin (13).

Studies to date indicate that acantholysis and phosphoprotein formation go together. In fact, the phosphoprotein content of an epidermal specimen is a reasonable measure of the degree of acantholysis as assessed histologically. Also, both acantholysis and phosphoprotein formation in epidermal slices are inhibited by drugs which inhibit pathways for the formation of ATP (4). While it is not yet possible to assign a causative role to these phosphoproteins in the production of cantharidin-induced acantholysis, it seems reasonable to suggest that the energy which is required in this form of acantholysis is used to produce ATP, which is then used in the phosphorylation of epidermal proteins by a protein kinase reaction.

In studies on experimental types of acantholysis it is appropriate to ask whether they have any biochemical relationship to the process as it occurs in disease states. One aspect of this study is that it suggests the chemical parameter of phosphoprotein phosphate as a means of comparing cantharidin-induced acantholysis and that of pemphigus vulgaris.

SUMMARY

Treatment of epidermal slices with disodium cantharidin resulted in a large increase in the formation of phosphoprotein, as demonstrated by phosphate-^{32}P labeling and chemical measurement of phosphate. The protein-bound phosphate decomposed to inorganic phosphate in 1N alkali, but the complex was stable in cold acid and dilute alkali and was not extracted with organic solvents. It precipitated with the fibrous epidermal proteins between pH 6.3 and pH 5.5. Protein from acantholytic epidermis contained up to 12 times as much phosphate-^{32}P as did protein from controls. The protein-bound phosphate-^{32}P represented from 17 to 30% of the total cellular phosphate-^{32}P. The

major organic phosphate in solution after enzymic and acid hydrolysis of the phosphoproteins was tentatively identified as O-phosphoserine.

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